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Growth, 1957, **21**, 73-88.

A NEW CYTOPLASMIC PATTERN IN STRIATED MUSCLE FIBERS AND ITS POSSIBLE RELATION TO GROWTH

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(Received November 19, 1956)

The questions of development, growth and regeneration, and certain problems concerning the general architecture of the contractile material in the various striated muscle fibers are still open for discussion. One of the most obscure points is the first layout of the contractile material in its essential pattern.

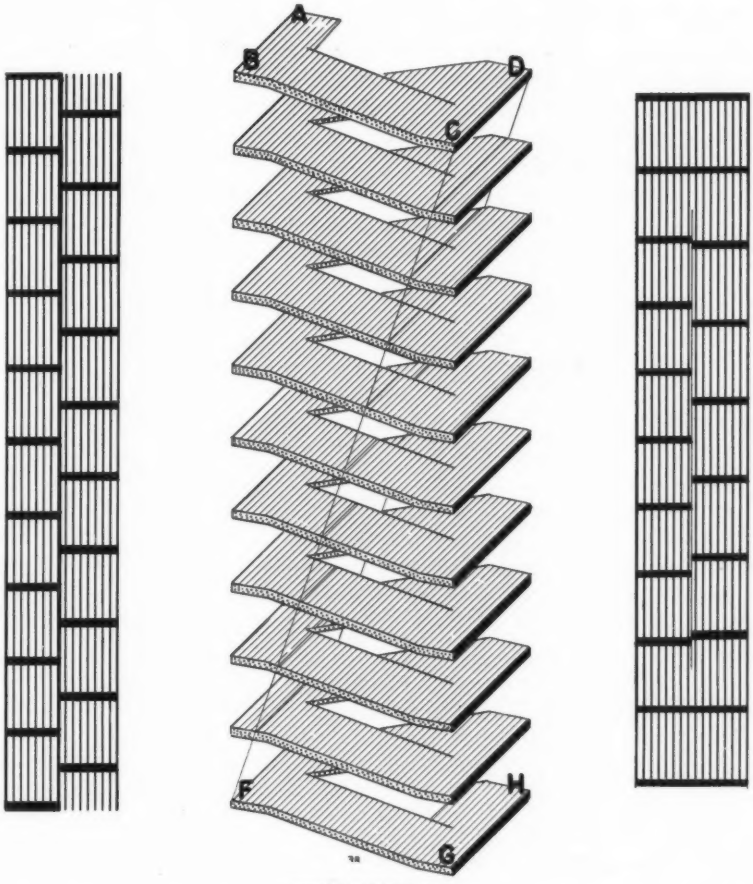
The fibrils develop from the apparently homogeneous protoplasm of the formative cell. Heidenhain (1898; 1911) considered the fibril bundles as derived from submicroscopic precursors, the "Molekularfibrillen" within the protoplasm. Other authors (Meves, 1909; Duesberg, 1910) advanced the idea that the fibrils arise from mitochondria. More recent support is given to Heidenhain's concept by D'Ancona and Fosi (1938), who found in several animals that the fibrils develop from longitudinally oriented lines of cytoplasm and that mitochondria only secondarily contribute to their formation. Similar fibril development from filamentous precursors and coalescence of granules have been described for several insect muscles (Tiegs, 1955); the granules were shown, however, to be different from mitochondria. Therefore the postulate that fibrils develop directly from mitochondria is now untenable (see Wassermann, 1929; D'Ancona and Fosi, 1938; Häggqvist, 1931; 1956; and Tiegs, 1955 for literature). Further growth in fibril width was believed by Heidenhain to occur by apposition. The growth of the total fiber in width then should occur by splitting of fibrils and apposition to the newly formed fibrils.

The concept of appositional growth inside the fiber finds support in the fact that muscle fibrils of newborn mice are thinner than fibrils of adult mice (Ruska, 1954). It has also been shown that one fibril may continue as two (Edwards and Ruska, 1955; Tiegs, 1955) which

might be considered as fibril multiplication by longitudinal splitting. It is, however, not conclusive whether this represents splitting or a synfibrillar arrangement of the contractile material (i.e., growth or architecture). Furthermore, not all muscle fibers contain bifurcating fibrils. High frequency fibers are characterized by the fact that the fibrils, at least in adults, never split or unite (Edwards, Ruska, Santos and Vallejo-Freire, 1956). Some fibers show frequent forking of the fibrils, as in heart muscle cells (Weinstein, 1954; Moore and Ruska, 1956). In other fibers a fibrillar arrangement cannot be found (Krüger, 1952; Krüger and Günther, 1956; Edwards, *et al.*, 1956). To this type, therefore, the question of apposition to individual fibrils and fibril splitting does not apply during growth.

Addition of sarcomeres in developing or regenerating fibers takes place at or near the muscle-tendon junctions or close to peripheral nuclei (Heidenhain, 1919; Speidel, 1938; D'Ancona and Fosi, 1938; Haas, 1950; Tiegs, 1955). When the distance the muscle has to contract is augmented experimentally, the muscle lengthens throughout the belly (Crawford, 1953). According to Heidenhain (1918), the length of the fibril should grow by transverse splitting of one muscle segment, and thus lead to a "Noniusperiode" (vernier type of band shift) where n segments of one muscle pillar meet with $n + 1$ segments of the adjacent pillar. Several such pillars, each adding a new segment, comprise what Heidenhain called a "Sphenode". However, it has been shown since that the Z planes ascend helically around a central longitudinal plane serving as axis (text fig. 1), and that the band shift results from the angle through which the axial plane is cut (Aurell and Wohlfart, 1936). Engelhardt (1955) further impressively demonstrated the helicoidal course of the sarcomeres on the fiber surface by rotating fibers under the light microscope. Matthaei and Tiegs (1955) even observed a spiral course of the contraction wave in muscle fibers with slow conduction and suggested transmission of excitation in the interior of the fiber along the helicoidal Z band.

A number of objections to the helicoid theory have been raised. Vernier effects could be distortions due to "shearing stresses or oblique tensions" (Jordan, 1933), and may be produced experimentally (Speidel, 1938). A discoidal arrangement, in which the segments are ordered like a pile of coins, has been reported in muscles of pycnogonids (sea spiders), insect gut muscles, higher insect flight muscles



TEXT FIGURE 1

Axial arrangement of the Z band in a muscle fiber with one central helicoid. The total fiber would extend considerably beyond the figure given in center. Sections parallel with or external to the planes CDGH and BCFG would give no band shift.

The figure on the left shows a section, parallel with CDGH, through the center of the axial plane leading to a non-vernier type of Z line displacement. The figure at right illustrates a section CDEF, through the axial plane resulting in an 8:7 shift. The extent of the shift depends upon the angle of cutting and the width of the plane. In the helicoid fiber the true sarcomere length is the shortest distance, i.e., where Z bands and filaments are normal to each other as in the planes ABEF and CDGH. The sarcomere lengths in shift areas are both greater than normal if one of the two sides is not cut parallel with the filaments. The right-hand figure gives the numbers but not true sizes of sarcomeres, due to projection of section.

(Tiegs, 1955) and tadpole tail muscle (Speidel, 1938). It is uncertain whether the discoidal fibers ever show vernier shifts. That exceptions to the helicoidal pattern exist is not surprising in view of the tremendous differences found in the structure of various muscles and fibers with different functional properties (Edwards, *et al.*, 1956).

The total experience derived from a great number of different muscles shows that (1) nonius periods and sphenodes are best explained by the direction through which one or more helicoids with central planes are cut, (2) several parallel helicoids within a single fiber increase the chances of finding vernier shifts and sphenodes, (3) in exceptional cases double helicoids occur around the same axis, (4) the turn is commonly to the left, and (5) the vernier shift is not generally believed at present to be correlated with growth.

Our own observations add a new aspect to the first layout of the muscle pattern, show a new possibility for the origin of non-vernier band shifts, and suggest increase of contractile material in the vicinity of nuclei.

Material and Methods

The muscles on which our findings are based are the *musculi latissimus dorsi anterior* and *posterior* of the thrush. The methods were the standard ones for electron microscopy, i.e., fixation with osmium tetroxide, dehydration with alcohol, embedding in methacrylates, and cutting with a glass knife. Observations were made with the RCA, EMU electron microscope, in the Department of Neuropathology of the Psychiatric Institute, College of Physicians and Surgeons, Columbia University, New York.

Observations

The more common arrangement of bird muscle fibers (Bennett and Porter, 1953) is that of the fibrillar type (tetanic or phasic), as all fibers of the *latissimus dorsi posterior* of many birds. This type is characterized by distinct fibrils, rarely uniting or splitting and by a well developed endoplasmic reticulum individualizing the fibrils (Fig. 1).

The less frequently occurring afibrillar type (tonic), such as the total *latissimus dorsi anterior* of the thrush (Krüger, 1950), is characterized by lack of fibrillization, and therefore a sparse longitudinal endoplasmic reticulum (Figs. 2-5). A large number of these fiber preparations from 5 birds showed scarcely one section without nonius periods. In the section shown in Figure 2, eight segments communi-

cate with nine, and five with six; and in Figure 3, two small opposing sphenodes are seen compensating the actual shift. The number of nonius to regular periods,* from the sarcomere where the shift begins to where the periods are once more in phase, varied from 2:1

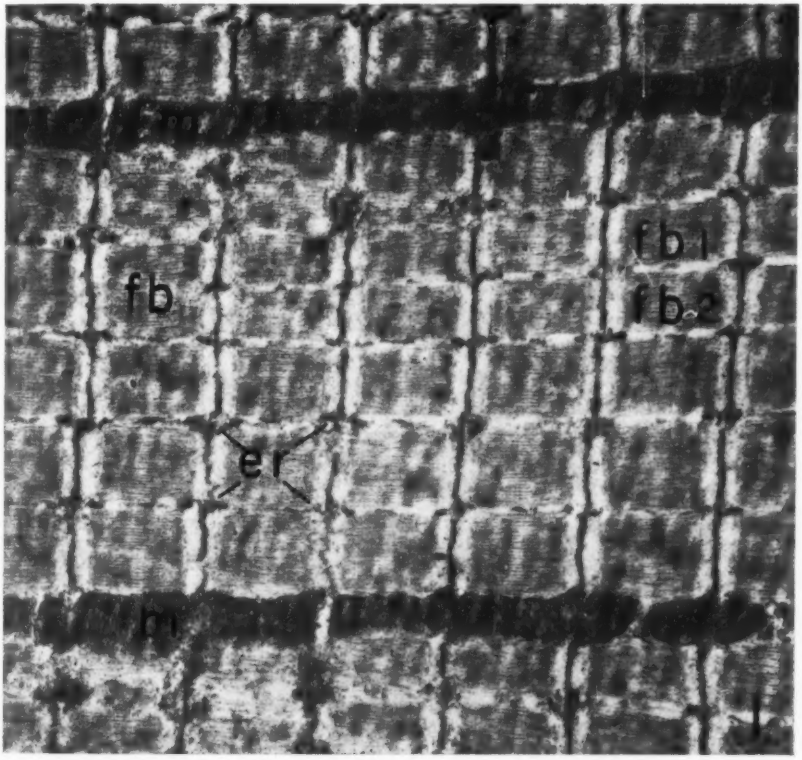


FIGURE 1

Longitudinal section through a fibrillar fiber of the *m. latissimus dorsi posterior* of a thrush. The fibrils are separated from each other by endoplasmic reticulum (er), which accumulates on both sides of the Z bands and around the middle of the sarcomeres. One fibril (fb) is splitting in two (fb1 and fb2). Chains of mitochondria (m) run parallel with the fibrils. (X 13,600).

* The use of the term vernier or nonius shift is conventional, but not correct if the shift results from a helicoid. In a true vernier shift there exist two different units on both sides of the shift. In a section through a helicoid with an axial plane and uniform pitch the shift is due to the direction of cutting but not to different units.

FIGURE 2

Longitudinal section through two afibrillar fibers of the *m. latissimus dorsi anterior* of a thrush. Note lack of discreet fibrils due to unsystematic separation of contractile material, and irregularity of Z bands. Section deviates from the direction of the mitochondria which have been found in other sections to form chains similar to those in the fibrillar fiber of Fig. 1. Two $n : n + 1$ shifts of striation are indicated in the left-hand fiber by the numbers in the sarcomeres. In the upper right, a portion of a more distant fiber from the same micrograph has been brought closer to be visible in the print. This fiber showed no shift but a period longer than the true sarcomere length since the filaments do not run in the direction of the sectioning plane. (X 15,400)

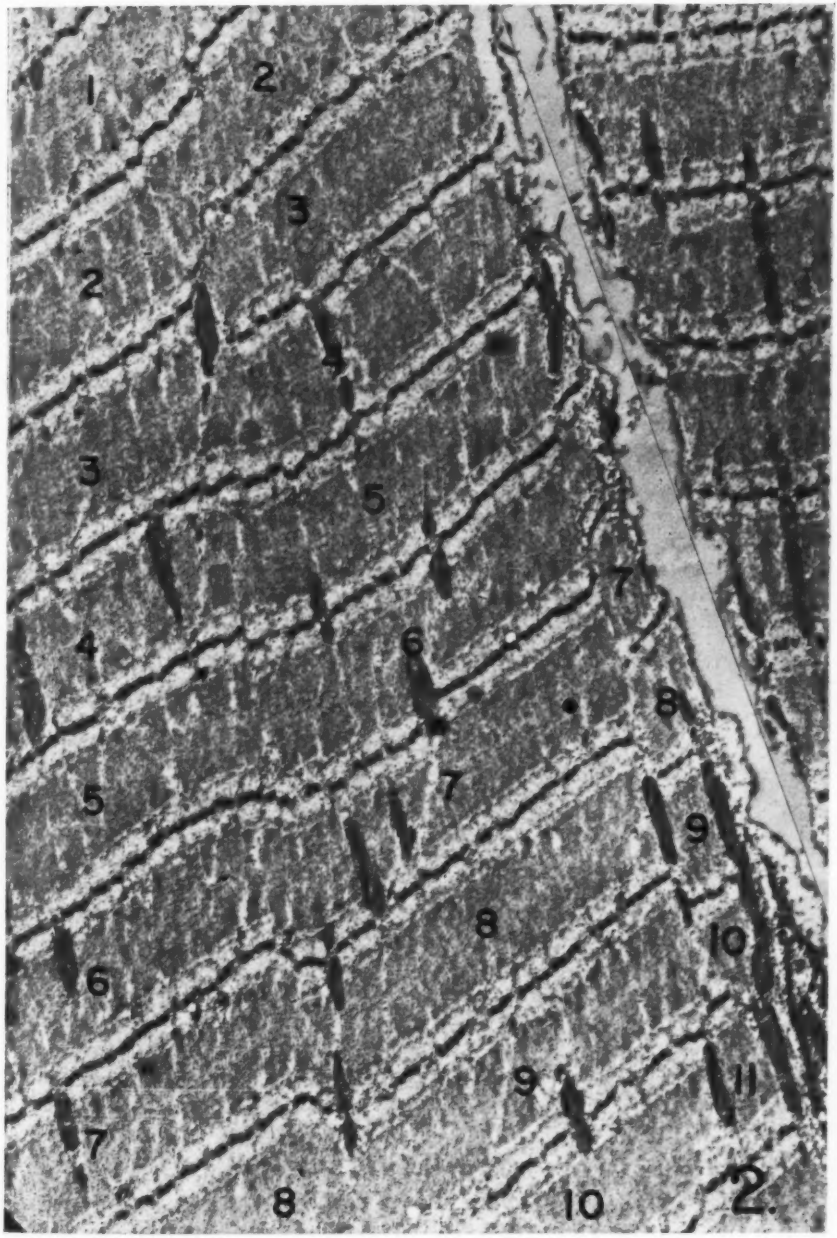

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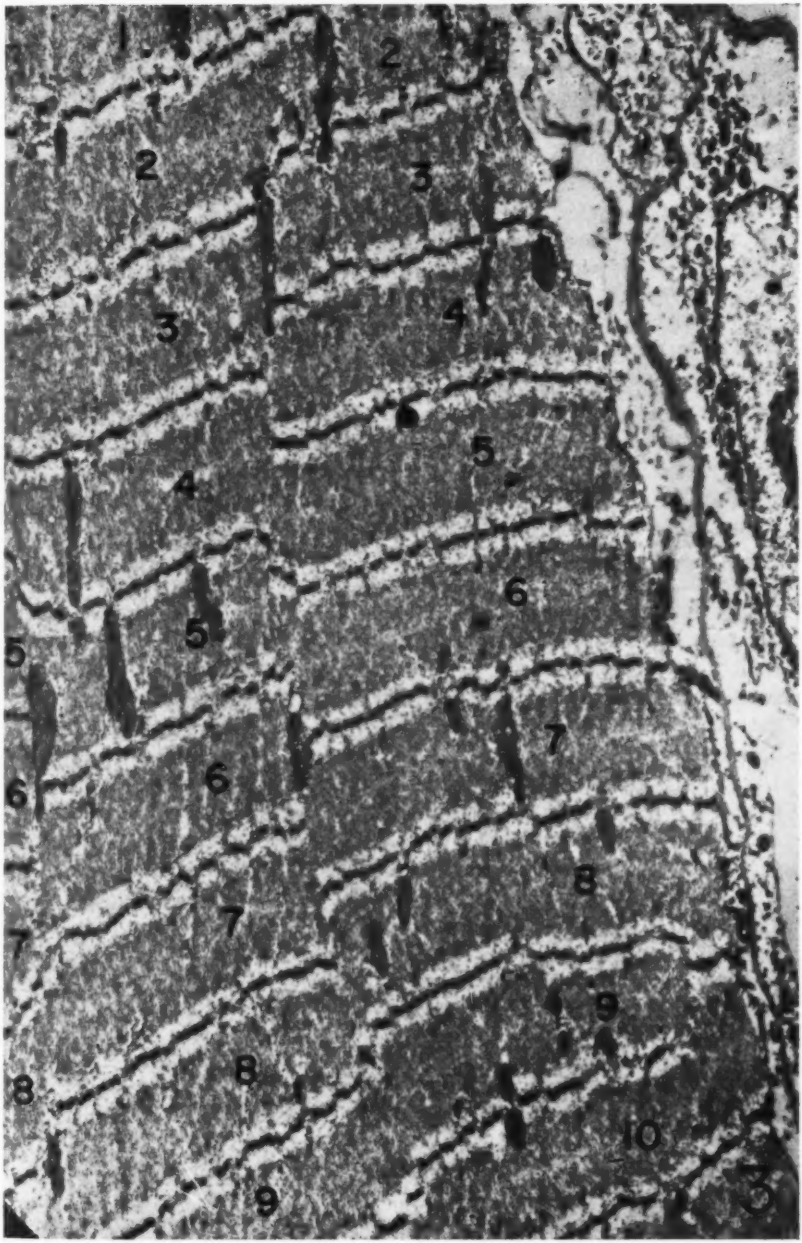
FIGURE 3

Longitudinal section of one afibrillar fiber of the *m. latissimus dorsi anterior* of a thrush. In center, two shifts are compensated over 9 segments. At left appears another shift where 4 segments communicate with 3. Shift lines are marked by sarcoplasm and mitochondria. Capillary at upper right. (X 15,400).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up to more than 11:10. The average ratio in 30 vernier shifts was 4.4:3.4. The nonius period columns could be recognized not only by shift in phase but also by their demarcations by sarcoplasm and mitochondria along the axial planes. The ratio was always n regular periods to $n + 1$ vernier periods. In many fibers there were several nonius periods, e.g., in a ratio of 3:2:1 giving the form of the sphenode. Splitting of segments should produce only one pair of shorter segments independent of the total number in a given vernier shift. Measurements of the Z to Z distances in the nonius segments gave no indication of splitting. The distances were in agreement with the helicoidal muscle structure, which leads to equal distances on both sides of the shift when the fiber is cut at an angle through the axial plane. (See Text Figure 1, diagram at right). The average regular distance along the shift was 1.68 μ; the nonius distance 1.59 μ. The true sarcomere length (where parallel to the filaments and where the filaments are normal to the Z bands) was 1.40 μ., as determined from those section areas which were parallel to the filaments.

A hitherto unknown pattern was observed peripheral to the muscle nuclei in fibers from five-month-old birds (Figs. 4 and 5). Two Z bands and an adjacent peripheral nucleus are seen in Fig. 4. In the cytoplasmic area between nucleus and sarcolemma appear several dark lines essentially oriented parallel to the Z bands but at a much shorter distance from each other. Between the lines may be observed faint filaments parallel to the myofilaments on the other side of the nucleus. Figure 5 illustrates a similar situation with a more complete pattern. Between the dark bands, which show a vernier shift, there appear more opaque and less opaque areas comparable to the A and I regions. A total of fifteen similar patterns, some of them extending over 4-5 μ in length and nearly 2 μ in width, have been observed in 10 different fibers. One fiber, cut secantially close to the fiber surface, showed the pattern on both longitudinal sides of the nucleus. The striations varied in number from 4 to 24, with an average of 10.4. The length of the period measured 0.090 to 0.172 μ, with an average of 0.128, the variations being greater than in final patterns. The appearance of these striations is strikingly different from that of ergastoplasm.

Discussion

The major problem under consideration in the present paper is the meaning of the vernier shift in relation to growth. Vernier shifts in

the final pattern of the muscle striation have been explained in two ways; either by splitting of one sarcomere in a muscle pillar to increase muscle length (Heidenhain-shifts), or by the direction of cutting through a helicoidal arrangement of the contractile material around a central plane (helicoid-shift; Aurell and Wohlfart). Both concepts lead to a relation of $n : n + 1$ segments along the shift, and both could be correct insofar as only this relation has been observed. However, Heidenhain's claim should lead to a single pair of shorter periods in the nonius, not resulting in a true nonius, whilst the helicoidal arrangement leads to equal distances of the Z bands over the full length of the nonius and a somewhat larger, but again equal, period on the adjacent side. Both should be longer than the true sarcomere length, whenever the cutting angle is not normal to the Z bands on one of the two sides. Since shorter pairs in nonius periods have not been found, our observations are strongly in favor of the helicoid structure.

The observation of a minute paranuclear pattern, similar in the appearance of cross bands and helicoid shifts to the classical muscle striation, leads us to the suggestion that this pattern is laid out by nuclear activity and represents the beginning of fiber growth in width, length, or both. The adaptation of the prodromal pattern to the final one then could give rise to striation shifts different from Heidenhain-shifts and helicoid-shifts.

At present the problem has the following status.

1. The short period pattern resembles the final fiber pattern in regard to regions and lines (A, I, and Z).

2. The helicoidal arrangement arises early in differentiation of contractile material, beginning with the 9-11 striation stages.

3. Short periods develop unilateral to the nucleus.

Prodromal patterns and helicoids in the thrush fibers signify an early layout of chemical and structural components. It is tempting to explain the unilaterality of the newly formed contractile material, in respect to the nucleus and the helicoidal arrangement, by rotation of one component around the other. A similar mechanism has already been suggested for the formation of the spiralized myelin layers around the nerve axon, i.e., rotation of the Schwann cell (Geren and Schmitt, 1955).

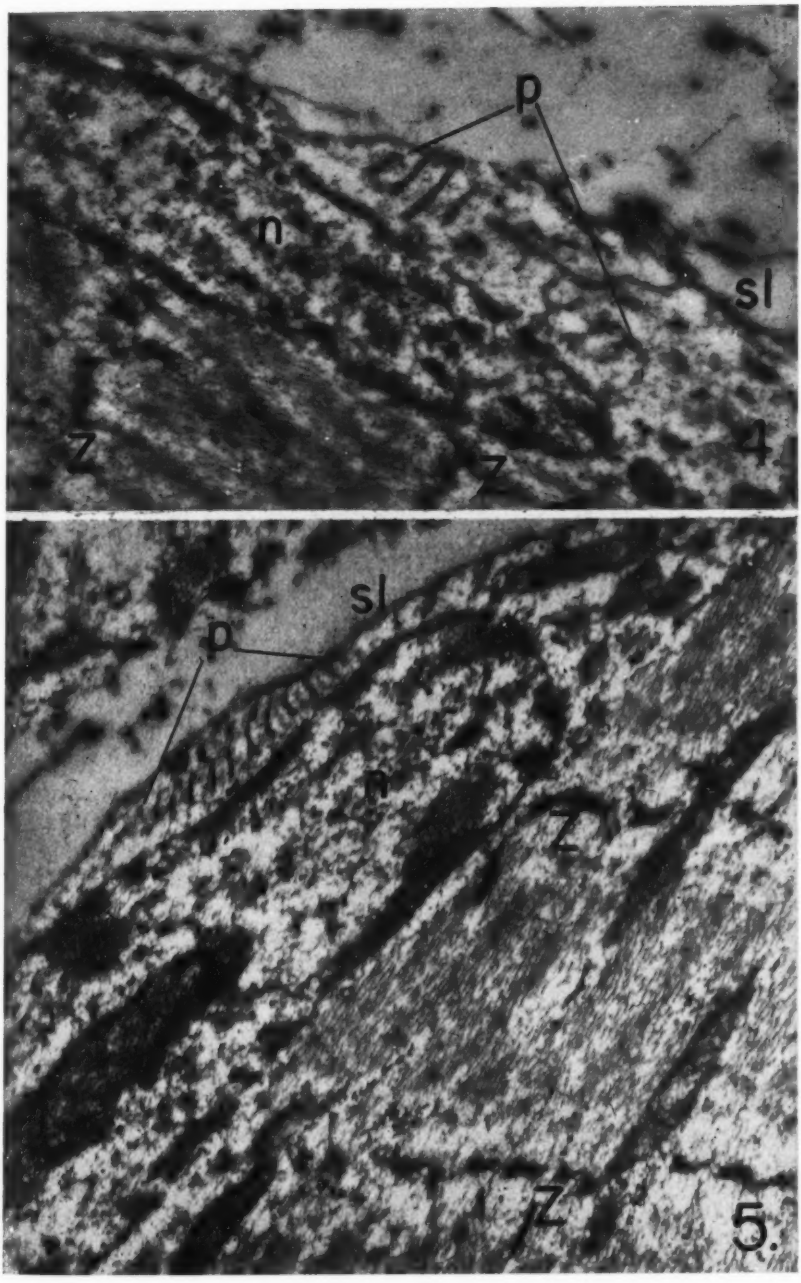
4. Intermediate periods between those of the prodromal paranuclear pattern and the final pattern were not observed.

FIGURE 4

Oblique section through *m. latissimus dorsi anterior* of a 5-month-old thrush. Small prodromal pattern (p) between nucleus (n) and sarcolemma (sl). Normal sarcomere length indicated by Z,Z. (X 25,200).

FIGURE 5

Oblique section through *m. latissimus dorsi anterior* of a 5-month-old thrush. Prodromal pattern (p) with apparent shift external to the nucleus (n). Dense Z, less dense A and light I regions visible in prodromal and normal patterns. (X 25,200).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Therefore, in our material, either we encountered only a given early stage of the pattern formation, or else a mechanism of splitting off single segments adapts the prodromal pattern to the final. Further experience is required to find whether intermediate periods do occur by simultaneous increases of segment length in the prodromal pattern. This would create growth shifts with $n : n + m$ bands between prodromal patterns and final patterns. Whatever the mechanism may be, the prodromal pattern seems to form a new muscle area, probably with its own nucleus, its own helicoid, and own cytoplasmic demarcation, thus, dependent on the position of the area, adding to the width and/or length of the fiber.

In general the growth of the muscle fiber includes 3 phases: (1) development (differentiation), (2) increase in size during growth of the individual, and (3) apposition by training. Related to these processes are regeneration and the continuous turnover of muscle substance. The total development and maintenance means the production of the specific substances (proteins, etc.), their arrangement into the specific pattern of the myomere, and the later addition to this pattern with increasing fiber length and width. All are generally considered as different mechanisms. But do we know whether these logical separations of different mechanisms are the ways taken by nature? It seems to be of primary importance to find out which steps or phases in the different processes include multiplication of nuclei and which do not. Nuclei accumulate in regenerating fibers and are involved in protein synthesis. Each nucleus of striated muscle fibers (Edwards, *et al.*, 1956), and of the muscle cells in the heart (Moore and Ruska, 1956) controls its own area by way of the endoplasmic reticulum. Separate mechanisms for growth in width and length, as discussed by Heidenhain and others, may take place in those phases of growth and training where the muscle pattern is present and increases without formation of new nuclear areas (Hoffman, 1947). Our concept is that, in growth and regeneration phases with multiplication of nuclei, the nuclei lay out new muscle patterns as visualized in this paper. The augmentation in length and width of the fiber would then occur in fields of specific nuclear activity.

Summary

A paranuclear pattern believed to be the prodromal pattern of contractile material is found in fibers of the *m. latissimus dorsi an-*

terior of young thrushes. In the same fibers, helicoid shifts occur frequently. The significance of these observations is discussed in relation to architecture and growth of muscle fibers.

ADDENDUM

Since this paper was submitted a similar paranuclear prodromal pattern has been observed in heart muscle cells of rat embryos.

(H. Fernández-Morán and H. Ruska, "Micromorphologic features of the embryonic rat heart-muscle": in preparation.)

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AN EFFECT OF RAUWOLFIA ALKALOIDS ON THE THYROID

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(Received June 3, 1957)

There are several reports in the literature on the antithyroid action of Rauwolfia alkaloids, but there is no agreement whether this effect is due to inhibition of the synthesis of thyroid hormone, to depression of the hypothalamus and of the hypophysis, or to neutralization of thyroxin in blood or tissue. The following experiments were undertaken in the hope of throwing light upon the mechanism of this antithyroid action.

Material and Methods

Seventy adult male rats of the Sprague-Dawley strain, weighing between 310 and 510 grams each were divided into four groups. Each group was kept in separate metal cages with screen bottoms to assure sanitary conditions, and were fed the same diet, consisting of Purina chow and tap water. Ten rats were used as controls, the other 60

TABLE I

Group	No. Animals	Experimental Conditions
I.	10	Control—I.P. saline daily, 42 days.
II.	20	Same as I + 1 unit TSH 48 hours before sacrifice.
III.	20	1.5 mg Rauwiloid in saline I.P. for 42 days.
IV.	20	Same as III + 1 unit TSH 48 hours before sacrifice.

received TSH* and Rauwiloid** in various amounts. In Table I the number of animals and the different experimental conditions are listed.

After completion of the experimental observations the animals were

* Thyroid stimulating hormone Thytropar® of Armour Laboratories, Chicago, Ill.
** Alkaloids of the Alseroxylon fraction of Rauwolfia serpentina. Rauwiloid® was furnished by Riker, Los Angeles.

anesthetized with nembutal and exsanguinated. The soft tissues of the neck anterior to the perivertebral fascia were removed in toto and fixed in 10 per cent formalin for histologic examination.

The functional state of the thyroids of the several groups was determined with radioiodine. One microcurie of carrier free I^{131} was injected intraperitoneally 24 hours prior to sacrifice. After fixation in formalin, each thyroid and trachea, in toto, was counted in a fixed geometry counter and the gamma activity was determined relative to a standard of the same shape, size, and position. An amount of I^{131} equal to the dose which had been administered to the animals was used as the standard. All determinations were made under identical statistical conditions. The results are given in Table II in terms of percentage of the dose which was administered.

TABLE II

Group	I^{131} % Uptake	Average Diameter	Average Height Epithelium
I. Controls (saline)	13.6	65 micron	7.7 micron
II. TSH	10.8	53.4 micron	11.5 micron
III. Rauwiloid	11.6	45.1 micron	10.7 micron
IV. Rauwiloid & TSH	11.3	41.8 micron	10.6 micron

Paraffin sections of the neck organs, 6 to 8 microns thick, were stained with hematoxylin and eosin. The measurements of follicles and cells were made in the same horizontal plane of all thyroids, i.e., cross sections were selected which showed the isthmus and the two upper parathyroids. The diameter of the thyroid follicles and the height of the follicle epithelium was measured in each gland with the micrometer eye piece. We selected the central portions of each lobe for these measurements because the peripheral follicles were much larger than those in the central portions.

Results

The average follicle diameter in the thyroids of the control animals injected daily for 42 days with saline was 65 microns, the height of the follicle cells was 7.7 microns and the colloid was well stained (Fig. 1).

The animals of the second group received one intraperitoneal injection of 1 unit of TSH and were killed 48 hours later. Their thy-

roids showed a slight decrease in follicle size, their diameter varied from 49.1 to 62.7 microns (average 53.4) and the epithelial cells were higher than in the controls, measuring 9.9 to 13.3 microns (average 11.5 microns). The colloid was thinner than in the controls, it

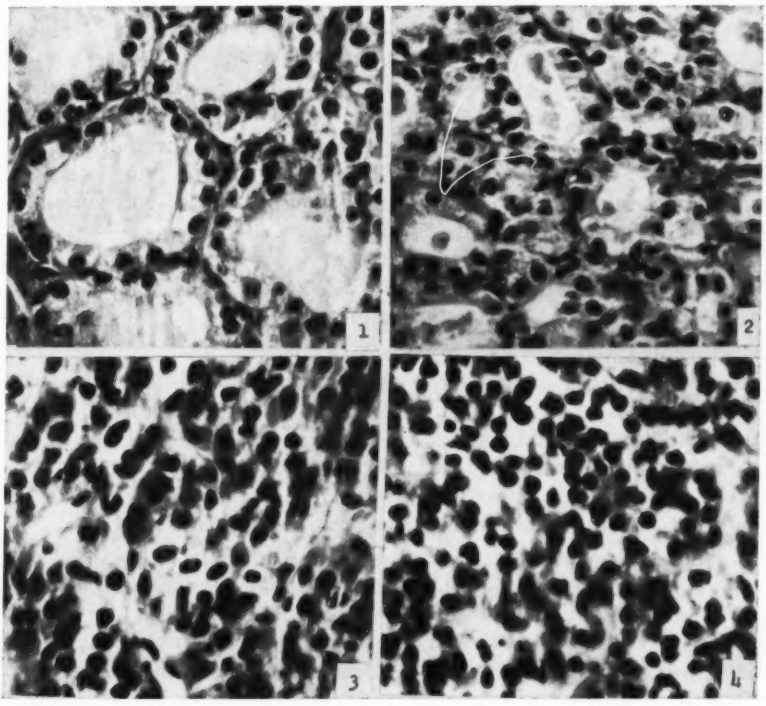

FIGURE 1
Thyroid of control animal. The follicles are large and contain well stained colloid. (x 640).

FIGURE 2
Thyroid of white rat which received 1.5 mg Rauwiloid per kg daily for 42 days. The follicles are small, the colloid is scanty and thin, the cells are high cuboid. (x 640).

FIGURE 3
Anterior lobe of hypophysis of control rat. (x 640).

FIGURE 4
Anterior lobe of hypophysis from the animal represented by Fig. 2. No evidence of atrophy. Some of the chromophobe cells are vacuolated as seen after thyroidectomy. (x 640).

was filamentous, stained pale red, and had many vacuoles. In the center of some follicles small bluish-red globules were noticed which gave a positive periodic acid-Schiff reaction.

The third group, consisting of 20 animals, received intraperitoneal injections of 1.5 mg of Rauwiloid® per kg daily for 42 days, with the same balanced diet as the control animals. The diameter of these glands varied from 35.4 to 56.6 microns, the average was 45.1 microns. The epithelial cells varied in height from 9.6 to 11.6 microns, the average being 10.7 microns (Fig. 2).

The fourth group received the same doses of Rauwiloid® for 42 days, but in addition one intraperitoneal injection of 1 unit of TSH was given 48 hours before sacrifice. The follicles of these animals measured between 39.15 and 43.5 microns, the average 41.8. The height of the follicle cells varied between 9.6 and 11.3 microns (average 10.6).

Discussion

Rauwolfia alkaloids caused definite histologic and functional alterations in the thyroids of our experimental animals. The disappearance of colloid, the decrease in size of the follicles, and the hypertrophy of the thyroid epithelium indicated an interference with the synthesis of colloid. The resorption of colloid appeared to be undisturbed, leading to exhaustion of the gland. Our findings suggest that Rauwolfia alkaloids act directly on the thyroid epithelium.

There is no histologic evidence in our experiments that Rauwolfia alkaloids depressed the function of the anterior lobe of the hypophysis (Figs. 3 & 4). Due to exhaustion of the colloid, the hypophysis probably increases its secretion of thyrotropic hormone. This would explain why the thyroid of our experimental animals did not take up more I^{131} after injection of TSH. As in lymphadenoid goiter, these thyroids were apparently under excessive stimulation by TSH and additional administration of TSH was without effect on the uptake of I^{131}.

Our results confirm the observation of Mayer, Kelly and Morton (3) (1956). These authors immersed slices of beef thyroid in a solution of Rauwolfia alkaloids and found a decrease in I^{131} uptake. They explained this phenomenon by blocking of the synthesis of thyroglobulin by Rauwolfia extracts. Our experiments do not support the view held by deFelice (2), Stern and co-workers (4) who assume a de-

pressing effect of Rauwolfia on the hypothalamus and hypophysis, or Bertelli's (1) conclusions that Rauwolfia alkaloids have a double action, namely a depression of the thyroid function and neutralization of thyroxin in the tissue.

Summary and Conclusions

Intraperitoneal injections of Rauwiloid® produced definite histological changes in the thyroids of white rats. The colloid disappeared, the follicles became smaller, and the epithelium increased in height. These findings suggest inhibition of the synthesis of thyroglobulin, while the colloid resorption is not disturbed.

The hypophysis of our experimental animals did not show signs of inactivity. The uptake of I^{131} by the thyroids of the animals receiving Rauwolfia was diminished and injection of TSH did not increase the uptake of I^{131}, probably because the exhausted thyroids were under increased stimulation by TSH.

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OÖGENESIS IN ADULT *DROSOPHILA MELANOGASTER*. II. STAGE DISTRIBUTION AS A FUNCTION OF AGE[1]

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The female fruit fly has remarkable synthetic abilities, since during the period of maximum egg production it ingests daily an amount of yeast which approximately equals its body weight and manufactures from the raw material a quantity of eggs which approximately equals 1/3 its weight (King and Wilson, 1955). The data to be reported provide a more precise description of the phenomenon of oögenesis with respect to growth rates than was available previously. They represent an extension of recent work (King, Rubinson and Smith, 1956) in which the process of oögenesis in the adult fruit fly, *Drosophila melanogaster*, was described and the development of the egg subdivided arbitrarily into 14 consecutive stages. Current data allow one to estimate the time required for an egg to pass through each developmental stage, and this information in turn allows the calculation of the relative rates of growth of the various components of the oöcyte. It is hoped that these data will serve as a basis of comparison for future studies involving mutations which in the homozygous state lower the fertility of females.

MATERIAL AND METHODS

The material and methods are identical to those described in the paper previously cited. The only facts which need to be added are that females were removed from culture bottles when 0-1 hours old. They were raised (without males) in groups of 25 on medium (for formula see King and Wood, '55 pg. 494) in half pint milk bottles at 25°C. Feulgen whole mounts were made of the ovaries of 0, 1, 2, 4 and 7 day old females.

[1] Research supported by the U. S. Atomic Energy Commission (contract No. AT (11-1)-89, Project 12) and by the Graduate School of Northwestern University.

Results and Conclusions

The data are presented in Table I. Since there are 12 ovarioles per ovary and therefore 24 per fly, one can conclude from inspection of these data that oöcytes in certain stages are represented approximately once in each ovariole. The oöcytes in question are: stage 1 (0, 1, 2, 4, 7 day flies), stage 2 (0, 1, 2, 4 day flies), stage 3 (0, 1, 2, 4 day flies), stage 6 (1, 2, 4, 7 day flies), stage 7 (1, 2, 4, 7 day flies) and stage 14 (7 day flies). The number of cells in stage 4 appears to remain fairly constant in the 0 through 4 day series. There is one stage 4 oöcyte per 1.26 ovarioles. The number of stage 5 oöcytes decreases linearly as the age of the female increases from 0 to 4 days. The number remains constant subsequently (one stage 5 oöcyte in every 5th ovariole). The number of stage 8 oöcytes remains constant at 1 per 2.5 ovarioles. Oöcytes are rarely found in stages 9 through 13. A few oöcytes in stages 9, 10 and 12 are found in each ovary of 1 and 2 day flies. Oöcytes in stages 11 and 13 are found in a fairly constant number (0.6 per female) in the 1 through the 7 day series. Stage 14 oöcytes are found in small numbers in 1 and 2 day flies, but are present in each ovariole after the 4th day. The ovaries of freshly hatched females are characterized by the absence of stages 8 through 14, and the proportions of oöcytes in stages 6 and 7 are lowered, so that they occur about as often as stage 4 oöcytes.

Normally fertilized females begin to lay eggs on the third day after eclosion (Wilson, King and Lowry, 1955). Egg laying reaches a maximum on day 4 and then remains fairly constant for about a week. It is known that the oviposition rate of virgin females is depressed, (ibid., Fig. 5 and Robertson and Sang, 1944, Fig. 2) and our data show a distinct shift in the stage distribution for the cells in the ovaries of 7 day virgin females. The number of oöcytes in stages 2, 3 and 4 is decreased below that normally found, and the number in stages 9, 10 and 12 is increased above that characteristic of 4 day flies. The total number of developing eggs per female remains constant ($\sim$180) in the 1 through 7 day series.

During the period of maximum oviposition females take roughly three days to lay 180 eggs. Here we assume a maximum oviposition rate of 60 eggs per day (cf. Fig. 5, Wilson, King and Lowry, 1955). Mated flies during the period of maximum egg production show a distribution of stages similar to that described for 4 day virgins. Since

TABLE I
Stage Distribution as a Function of Age.

Age of Virgin female (days)	Number of ovaries observed	Avg. total developing eggs per female in stage ± ($s_{\bar{x}}$)														Total developing eggs per female	Avg. developing eggs per ovariole
		1	2	3	4	5	6	7	8	9	10	11	12	13	14		
0	13	24.8 (1.7)	25.1 (2.3)	22.2 (0.8)	20.4 (1.5)	14.5 (1.4)	20.2 (1.6)	18.9 (1.4)	0	0	0	0	0	0	0	146.1	6
1	18	24.5 (1.3)	24.9 (0.9)	25.0 (1.1)	20.4 (1.6)	11.8 (1.4)	27.4 (1.1)	25.6 (1.1)	11.5 (1.1)	3.1 (0.7)	2.7 (0.8)	0.5 (0.2)	4.5 (1.8)	1.8 (0.5)	2.6 (0.5)	186.3	7.5
2	10	24.5 (4.2)	25.6 (3.3)	24.6 (2.4)	19.2 (1.7)	9.4 (1.0)	24.2 (1.7)	25.0 (1.7)	11.2 (1.0)	9.2 (1.4)	5.2 (1.0)	0.6 (0.2)	3.2 (0.4)	0.6 (0.2)	1.8 (0.5)	184.3	7.5
4	17	25.0 (2.2)	22.6 (2.0)	30.0 (2.9)	18.4 (3.1)	4.9 (0.2)	22.6 (1.6)	22.0 (1.7)	9.0 (0.2)	1.7 (0.2)	0.6 (0.2)	0	0.1 (0.03)	0.1 (0.03)	17.1 (1.6)	174.1	7.1
7	10	24.7 (1.5)	20.2 (2.4)	18.4 (2.0)	11.8 (1.8)	5.8 (1.0)	23.8 (6.1)	23.4 (2.8)	12.0 (1.6)	7.2 (0.7)	8.4 (1.7)	0.8 (0.4)	1.2 (0.6)	0.4 (0.4)	23.6 (3.2)	181.7	7.4

at any given time during this period there exist 180 eggs in the ovaries, it follows that a steady state is maintained and that it takes approximately three days for an egg to pass from stage 1 to stage 14. One egg would complete ovarian development each 0.4 hours on the average. A given ovariole would complete synthesis of an egg each 9.6 hours, and therefore each oöcyte in an ovariole would be less mature (by 9.6 hours) than the oöcyte immediately behind it. We assume that ovarioles 1 through 24 each produce a mature egg before ovariole 1 produces a second egg (cf. Figure 1). The amount of time spent in a given stage should be proportional to the number of eggs found in that stage. For example, if stage 4 oöcytes make up 10% of the total developing eggs, then stage 4 should take 7.2 hrs. (10% of the developmental period of 72 hrs.). It thus becomes possible to estimate the developmental period for each stage (cf. Table II) and to convert Figure 1 of the earlier paper on oögenesis (King, Rubinson and Smith, 1956) to Figures 2 and 3 of this paper.

Freshly hatched females contain substantial numbers of stage 7 oöcytes. It is known (cf. Table II) that a stage 7 egg can complete development in about 14.5 hours. However, mature eggs are not produced in substantial quantities for a period of at least 48 hours. The cause of this delay is unknown.

TABLE II
The Stage Distribution and Time Spent in Various Stages for Oöcytes of Females During the Period of Maximum Oviposition.

Stage	% of developing eggs found in this stage	Time (hrs) spent in stage
1	13.3	9.58
2	13.3	9.58
3	13.3	9.58
4	10.6	7.62
5	2.8	2.01
6	13.3	9.58
7	13.3	9.58
8	5.2	3.74
9	1.0	0.72
10	0.3	0.22
11	<0.1	<0.07
12	<0.1	<0.07
13	<0.1	<0.07
14	13.3	9.58
	100.0	72.00

The values in column 2 are calculated from the data for 4 day virgins presented in Table I. The value given for stages 1, 2, 3, 6, 7 and 14 represents the average of the lumped data for these 6 stages.

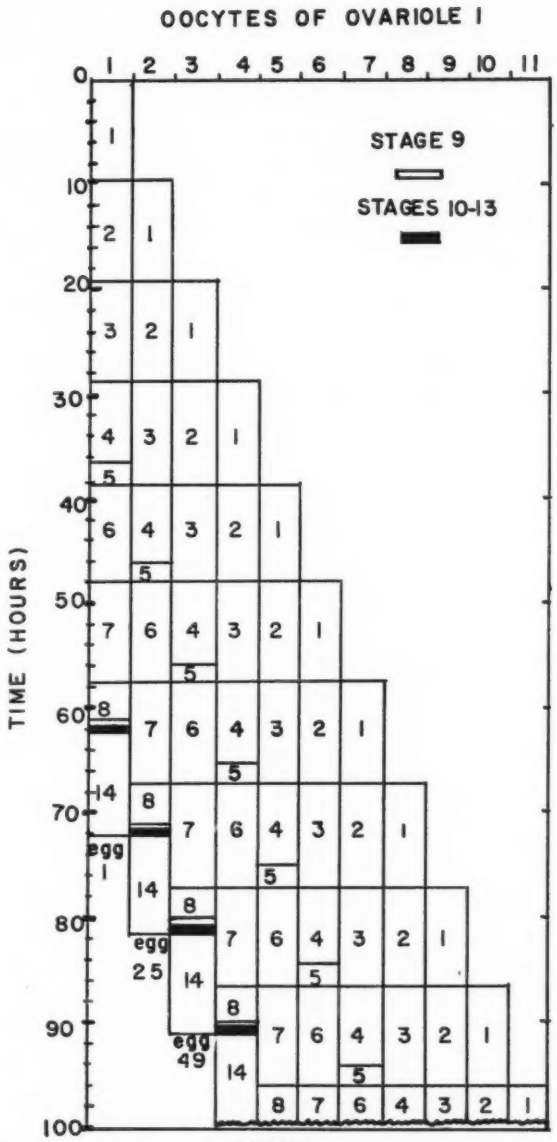

FIGURE 1

A model which describes the distribution of *Drosophila* oöcytes as a function of time for a given ovariole. Thus the ovariole will contain oöcytes in stages 1, 2, 3, 4, 6, 7 and 14 74 hours after oöcyte 1 begins development. Oöcyte 2 occupies the most posterior chamber once oöcyte 1 is laid. Ovariole 1 produces eggs 1, 25, 49, etc.; ovariole 2 produces eggs 2, 26, 50, etc.; while ovariole 24 produces eggs 24, 48, 72, etc.

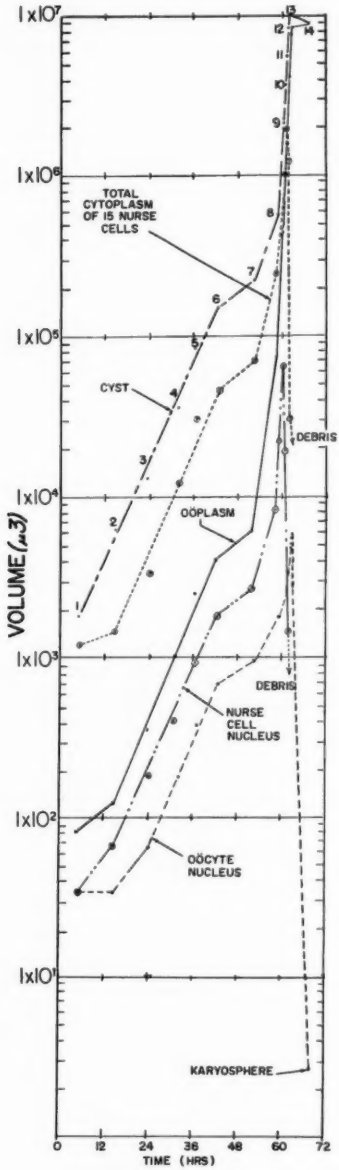

FIGURE 2

Change in volume as a function of time during oogenesis in *Drosophila melanogaster*.

An analysis of Figure 2 shows that growth of the various components of a developing egg during stages 2 through 6 (a time interval of approximately 38 hours) appears to be exponential. An average time of approximately 7.6 hours is required to double the volumes

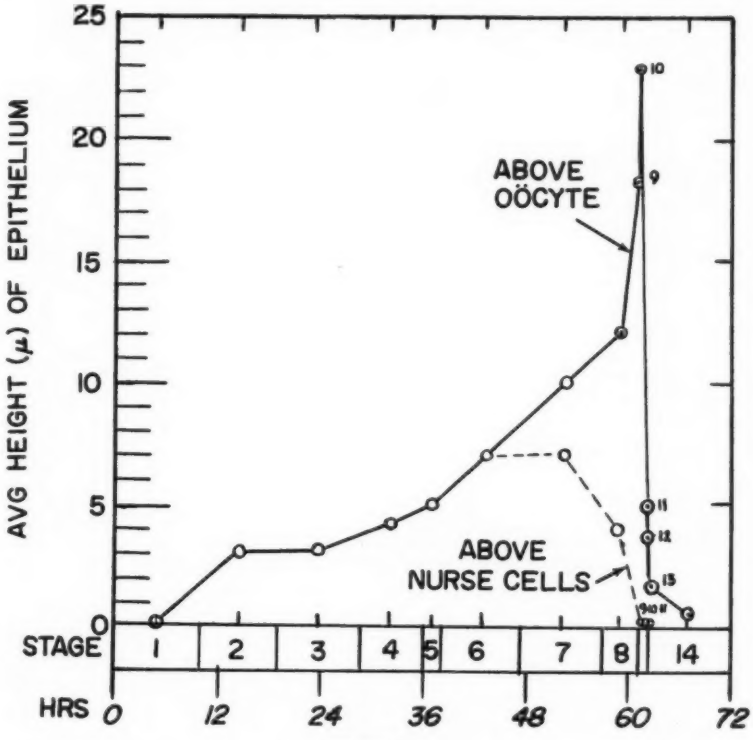

FIGURE 3

Change in height of the epithelial layer as a function of time and stage during oögenesis in *Drosophila melanogaster*.

and consequently a 32 fold increase occurs in the volume of the cyst, the oöcyte nucleoplasm and cytoplasm, and the nurse cell nucleoplasm and cytoplasm during this interval. During stage 7 the chamber elongates and there is a decrease in growth rate. Next (stages 8 through 10) there is a rise in the rate of volume increase

for the nurse cell cytoplasm and nucleoplasm. The doubling time is now approximately 1.5 hours. During this 4.7 hour period (cf. Figure 2) the epithelium above the oöcyte increases in height by at least 11 micra. During stages 11 and 12 the nurse cells shrink and are subsequently resorbed. The oöplasm undergoes a 128 fold increase in volume during stages 8 through 13 (a time interval of about 5 hours). The doubling time is 0.7 hours. Thus during the period of yolk production (stage 8 through 13) the rate of oöplasmic volume increase rises by a 10-fold factor.

Summary

The stage distributions of oöcytes in the ovaries of virgin 0, 1, 2, 4 and 7 day old adult *Drosophila melanogaster* are described. Estimates are given for the time required for an egg to pass through each developmental stage. Relative rates of growth are calculated for the various components of oöcytes.

Acknowledgments

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THE DIRECT AND INDIRECT EFFECT OF SHORT-DISTANCE X-IRRADIATION ON THE MITOTIC ACTIVITY OF THE RAT EPIDERMIS AND HAIR FOLLICLES[1]

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This work was undertaken with the object of studying the effect of x-irradiation on the skin of growing rats and determining whether irradiation stimulates or inhibits the mitotic activity in portions of skin other than those locally treated. This question arose, because in previous studies x-irradiation had been found to cause diminution, not only of an irradiated but also of a nonirradiated, tumour in the same experimental animal (24, 25). A corresponding decrease was noted in the mitotic activity (24). Irradiation energy has been assumed to produce tumour-inhibiting growth-factors (24, 25). Similar observations were made by Lorenz (17), and he spoke of the distance effect of irradiation energy on the tissues. He obtained this effect with doses smaller than the tumour dose. Kepp and Hoffmann (14) irradiated two fields on the tail of mice at an interval of 3 days. Skin erythema and the resulting necrosis were more powerful and appeared earlier in the area irradiated later. Thus treatment of the first field had shortened the period required for a reaction in the second field. This was stated to be a form of indirect radiation effect of humoral or of nervous and reflex origin. Hoffmann (12) produced necrosis even more rapidly in the skin of a rat's tail when irradiating the second field at an interval of 15 days. He attributed this chiefly to a humoral effect.

The present study of the distance effect of x-irradiation is concerned with the same basic problem, i.e., whether actin energy affects the control of cell division. To answer this question we chose to

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study the possible mitotic variations in the epidermis and hair follicles of the rat following x-irradiation.

Material and Methods

Eighteen white male rats of the Sprague-Dawley strain and 18 female rats were used for the experiments; they were all 3 months old. The animals were divided into 6 groups, each including 3 males and 3 females. The 24 rats divided into 4 groups were given short-distance x-irradiation with the Siemens-Monopan equipment of the Central Institute of Radiotherapy (60 kW, 4 mA, distance 1½ cm, radiation field 2 cm^2).

Short-distance x-irradiation was administered at 4 p.m. on three successive days to the same area of the interscapular skin of the animals in such a manner that the skin to the left of the midline was pulled as much as possible outward from the body, the mouth of the tube touching this skin, so that x-rays could not reach other parts of the rat's body (Fig. 1). Thus, the area to the right of the midline, symmetrically opposite to the irradiated field, also received irradiation but the rays were oppositely directed. The single dose was 1000 r and the duration of the treatment 39 seconds. The total dose for each experimental animal was 3000 r. The 12 rats serving as controls in the two groups received no irradiation.

Six hours after termination of irradiation treatment the 6 rats of the first group were decapitated. The rest were decapitated as follows: Irradiated group II and control group I two days after the end of treatment, irradiated group III on the seventh day, and irradiated group IV and control group II, 14 days after treatment. From all experimental animals biopsy specimens of skin were removed from (a) the irradiated field, which was definitely outlined, (b) the opposite area of the interscapular skin, (c) the gluteal region, (d) the left fore-leg, (e) the abdomen in the midline, and (f) the middle portion of the tail. The specimens were fixed in Bouin's solution and the sections stained with hemalaun-eosin.

Counts of mitoses were made by calculating separately the mitotic figures per 2000 cells according to a method previously described (23). The mitoses in the hair follicles were counted only in groups a, b, d and e.

The statistical significance of the results was tested by Student's t-test (10).

RESULTS

A. *Mitotic activity of epidermis.* The mitotic activity in the epidermis of the control rats and the irradiated rats is shown in Figure 2 calculated as averages of all six animals of every group. The mitotic index of the controls was fairly constant in all groups (a, b,

FIGURE 1

The method of x-irradiation. The skin of the interscapular region of the rat was pulled outward from the body, and the mouth of the tube was touching the skin to the left of the midline. The area to the right of the midline thus received also irradiation but the rays were oppositely directed.

c, d, e and f), i.e., 3-4 mitoses per 2000 cells. Five mitoses were exceeded only in group f.

In the irradiated rats, the fluctuations in mitotic rates were similarly directed and quite considerable. After x-irradiation the mitotic index was low, 1.0 ± 0.37 (mean ± standard error). It then increased continuously, and one week after termination of treatment it was 8.2 ± 1.5 in group a and 6.5 ± 0.55 in group b. One week later the mitotic index in both these groups equalled that of the controls.

In group c, in which the specimens were taken from the back of the rats, thus from a non-irradiated area, the mean mitotic index immediately on termination of treatment was 8.0 ± 0.54, i.e., significantly above the level of the controls statistically ($P < 0.001$), and after two days it had already returned to the level of the controls where it remained throughout the experiment. The same was noted in group d, the skin of one fore-leg. Six hours after last irradiation the mitotic index was 7.5 ± 0.83, what is clearly above normal level, the difference from normal being statistically significant ($P < 0.01$). In group e there was a smaller rise after treatment, mitotic index 5.6 ± 0.79, the difference from normal being significant with $P < 0.05$, but the curve then remained at the level of the control rats. In group f the mitotic index fluctuated between 3 and 6, showing no significant variance.

B. *Mitotic activity of hair follicles.* The mitotic index in the hair follicles of the skin was higher than in the epidermis in the control rats. In all groups it was about 10-14, thus its level was about similar in all parts of the skin studied. In the irradiated areas, i.e., in groups a and b, where it was lowest immediately after treatment, it had increased after one week in group a from 6.0 ± 1.1 to 19.2 ± 1.2 and in group b from 6.7 ± 0.62 to 18.0 ± 1.3, the differences being in both cases significant as compared with the level of the controls (a = 13.0 ± 1.5, b = 12.7 ± 1.1, $P < 0.02$). One week later the mitotic index in these groups corresponded approximately to that of the controls. In groups d and e, the mitotic indices obtained throughout the experiment were about the same as in the control groups.

Discussion

The mitotic indices in the epidermis of the controls were relatively uniform in all the specimens studied, varying on an average from

3 to 4 in 2000 cells. The mitotic activity in the skin of rats of varying age has been studied previously by e.g. Hanson, who found it to vary from 0.76 to 2.6 per cent in specimens from different parts of the body (11). Andrew and Andrew counted 0—3 mitoses per 1000 cells in the stratum germinativum (1). Teir, Kiljunen and Putkonen found that the average mitotic activity in the back skin of 6-week-old rats was 2 per thousand (22). The total mitotic activity obtained by Kiljunen (15) when studying the epidermis of rats of varying age was 1.47 ± 0.77 per thousand; this figure being slightly lower than ours, but those reported by the already mentioned authors are of the same order of magnitude as our values.

On the basis of our study the mitotic activity does not vary from one part of the body to another in normal animals—an observation which is entirely in accord with those of Loeb and Haven (16), Carleton (4) and Kiljunen (15).

In the portion of the skin receiving direct irradiation the mitotic activity recorded 6 hours after the end of the last treatment was considerably lower than in the control material. Two days later the mitotic activity was still below that of the controls. One week after the last treatment it had increased considerably, the average being 8.0 ± 1.5 mitoses (Fig. 2), this was followed by a fall and after two weeks the mitotic index was normal. This seems to be an example of a case of a typical roentgen effect in which the amount and time of the drops and rises in mitotic activity are determined by the dose and its fractionation. Similar variations in mitotic activity have been noted by Spear (21), though he used lower dosages and a single irradiation. For this reason he had no appreciable tissue injuries in the irradiated area and the compensatory increase in mitoses therefore occurred much earlier, about 4-6 hours after irradiation. However, the dose used by us (1000 r 3 times) resulted in distinct radiation damage, which seems to account for the fact that the compensatory rise was found considerably later, the maximum occurring between the second and seventh day and the mitotic rate being considerably more than double the normal as late as on the seventh day.

The epidermis on the side opposite to the irradiated area received 50 per cent of the skin dose administered. The mitotic activity here shows similar but somewhat slighter variations, as would be expected.

As indicated by Figure 2, the variations in mitotic rate have a completely different rhythm in the non-irradiated area: 6 hours after the

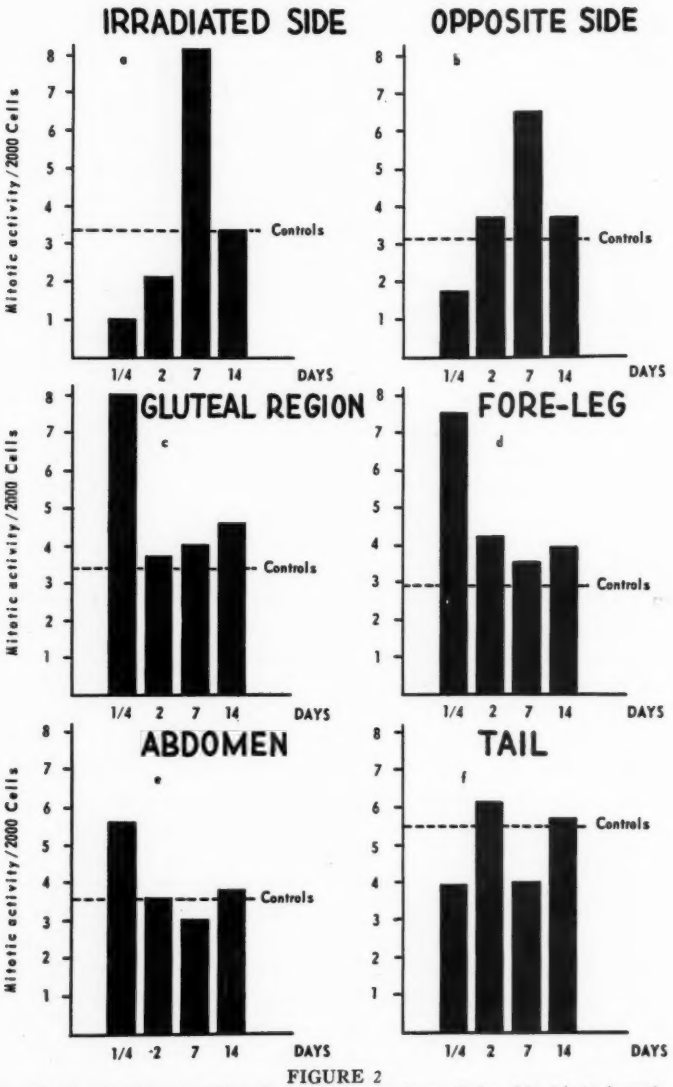

FIGURE 2

The mitotic activities of epidermis in various regions of the skin ¼ (6 hours), 2, 7, and 14 days after the last of three in successive days given x-irradiations, every single dose being 1000 r. Black columns = mean mitotic activities/2000 cells in the experimental animals. Fragmentary lines = mean level of mitotic activities/2000 cells in the control animals in corresponding regions of the skin.

last treatment the index was clearly above normal. This is quite contrary to the findings described above for the irradiated areas. Restoration to the normal level seems to have occurred within two days from the last treatment. The variations in the tail were of considerably less extent.

The question arises as to whether the non-irradiated areas mentioned had been exposed to secondary radiation. This seems to have been impossible: during short-distance irradiation the tube was in direct contact with the irradiated area and the beam of rays was limited to the area of exposure. Thus the cause of the phenomenon must be sought elsewhere.

A similar phenomenon has been reported by Pfuhl and Kuhtz (20) who studied the effect exerted on the mitotic activity in connective tissue of rabbits by 800 r administered as a single dose with a Cu-filter to a definitely limited area of the back skin. They observed mitoses to occur in the subcutaneous connective tissue also in the shielded area, and stated that the only possible explanation is a humoral distance effect. Luther (18), however, did not agree with the above investigators.

Several other observations are in favour of some kind of distance action. Van Dyke and Huff (8) studied parabiotic rats, irradiating the skin of one rat with 900 r with the purpose of causing epilation, the other rat being shielded with a lead plate. About 4 weeks after the irradiation, epilation was observed in both rats, and in the non-irradiated animal it was even more pronounced. This can be explained only by humoral effect.

Caspari, on the other hand, expressed the view (5) that, in connection with tissue destruction, so-called necrohormones which stimulate cell division when present in small amounts and arrest growth when present in large ones, might be formed or liberated. In tissue culture of animal tissue Fischer (9) observed rapid growth after the cultures had been subjected to injury. Cook and Fardon (7) observed a quantitative effect: slight injury stimulated growth while serious injury often arrested it. It seems obvious that the tissue destruction caused by irradiation in our study is relatively slight though even objectively clearly demonstrable. If being so, it may be assumed that products of tissue destruction are liberated from the destroyed area, which, through humoral spread, cause a rise of mitotic activity in other parts of the body. Further studies are required to determine whether this

effect is limited to the skin, i.e. to homologous tissue, or whether it extends also to other organs.

Studies of the hair growth in various experimental animals have been reported by several observers but few have been concerned with the mitotic activity in hair follicles. Kiljunen reported the mitotic activity in hair follicles of 3-week and 1-year-old rats as 1.62 ± 0.65 pro 1000 cells and 1.47 ± 0.65 respectively and of 6-week-old rats as 9.98 ± 0.65 (15). Thus, in the former groups the follicles were resting, in the latter they were growing. The values obtained by us are slightly higher, the average varying in the control material from 9 to 14. The wide variation is probably accounted for by variations in the stage of the hair growth cycle.

The fact, that 6 hours after the last treatment, the mitotic activity in the irradiated area and on the side opposite to it, was considerably below the control values in our study too, seems to be due to a natural radiation effect. As in the epidermis a compensatory rise above the control level followed also in the hair follicles one week after the end of the treatment, and by the time the next biopsy specimen was removed it had again returned to the level of the controls. The powerful rise in mitotic activity observed in the non-irradiated area of the epidermis 6 hours after termination of treatment was not noticed in the corresponding areas of the hair follicles: the mitotic indices in the hair follicles of the non-irradiated fore-leg and the abdominal skin were similar in the experimental and in the control groups. Whether a rise occurs at some time during the first week is difficult to evaluate on the basis of the method used here.

It is thus evident that the epidermis reacts more sensitively than the hair follicles to the variations induced by local x-irradiation in the mechanism controlling the mitotic activity of the skin. This observation, as well as the mitotic counts on individual specimens, seems to support Kiljunen's statement (15) that cell division in the epidermis and hair follicles is at least to some extent subject to different sensitivity. Chase et al. (6) studied the hair growth cycle and noted that in growing follicles, or in so-called anagen, the mitotic activity in the epidermis is increased and the epidermis thickens. In the resting phase or telogen, the hair follicles are short, the epidermis is thin, and in both there is low mitotic activity. Argyris found that succinic dehydrogenase activity increases in growing follicles, the epidermis also showing increased enzymatic activity in the early part of the

hair cycle, when the epidermis is undergoing hyperplasia (2). During the resting phase of the cycle the epidermis shows low succinic dehydrogenase activity. The unspecific esterase activity is well marked in the resting phase and decreased in growing follicles. Argyris assumes that powerful succinic dehydrogenase activity is related to intense cell division and participates in the Krebs cycle in producing oxydative energy, which is absolutely necessary in cell division in the skin as demonstrated by Medawar (19) and Bullough (3). According to Argyris' studies, the mitotic activity of the epidermis is correlated with that of the hair follicles in the early part of the hair growth cycle. His enzyme studies showed no distinct correlation later in the cycle. He did not carry out counts of mitoses, and thus directly comparable data are not available.

It seems that the doses of short-distance x-irradiation used by us are able to produce variations of fairly long duration in the mitotic activity of the rat epidermis and hair follicles of the irradiated area. In addition in the epidermis a distinct stimulation of mitosis was found in several places distant from the irradiated area and this stimulation appeared on termination of treatment. This must be interpreted as a kind of indirect or distance action of irradiation, which probably can be transmitted humorally or as pointed out by Jolles (13) as action on molecules not directly ionized or excited, but which are exchanged as a result of absorption of radiation energy which has excited or ionized solvent molecules, giving products which then act as energy carriers.

Summary

1. This report is based on studies of 36 rats, 3 months old, divided into six groups. 24 rats divided into four groups were irradiated with short distance irradiation, a daily dose of 1000 r for three successive days. 12 rats served as controls. The area of irradiation was in the interscapular region to the left of the midline, the skin being pulled out from the body so that an area symmetrically opposite on the right side of the midline also was irradiated. On termination of treatment biopsy specimens of skin were removed from the irradiated areas and from different parts during a period of two weeks.

2. The mitotic activity in the epidermis of the irradiated area was considerably below the control level after 6 hours, and significantly above it after one week. In the non-irradiated gluteal area and in

the skin of the left fore-leg the mitotic indices were significantly higher than in the controls 6 hours after last treatment. The abdominal skin showed a slight rise and the skin of the tail a still slighter rise. After one week these mitotic indices were at the normal level, and the same applies to the figures for all groups after two weeks. The mitotic activity in the hair follicles was calculated from the irradiated areas, from the left fore-leg, and the abdominal skin. In the irradiated areas the mitotic index was below normal on termination of irradiation; after one week it was significantly higher than normal, but after two weeks it had returned to normal again. In the fore-leg and the abdominal skin no significant fluctuations were observed in the hair follicles.

3. The mitotic rate in the epidermis of the non-irradiated area has a different rhythm after irradiation. The phenomenon can be accounted as being due to humoral distance action.

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THE EFFECTS OF GROWTH HORMONE ON PHENYLALANINE-TYROSINE DEFICIENT AND PAIR-FED RATS*

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In a series of experiments dealing with the histopathological effects produced in rats fed a purified diet totally lacking in a single essential amino acid, one of the most consistent responses was that of pronounced dimunition in cell size and cytoplasm of the anterior pituitary acidophils. These reports suggested that the total omission of dietary phenylalanine (26), histidine (27), tryptophan (28), isoleucine (29) or threonine (30) directly affected the pituitary acidophils resulting in a loss or reduction of growth hormone secretion and that this lack of adequate endogenous growth hormone was responsible for the cessation of body growth as expressed by the inactivity of the proximal epiphyseal cartilage. In these same experiments, pair-fed control rats showed that a hypocaloric diet was capable of producing somewhat similar, but very much less severe, effects. That caloric restriction is capable of causing reduction in skeletal growth is in agreement with the reports of Handler, Baylin and Follis (14) and Saxton and Silverberg (25).

Numerous experiments have been described wherein anterior pituitary growth hormone administration failed to produce weight gain in rats deficient in riboflavin (4), pantothenic acid (4, 17), choline (13), essential fatty acids (6), vitamin A (8) or pyridoxine (3). Bavetta *et al.* (2) were unable to obtain significant differences in body weight gain between tryptophan-deficient rats treated with growth hormone and their saline-treated controls. However, they made the important observation that, while growth hormone failed to increase the body weight, it caused bone growth and dentin formation to be resumed.

Chow and Greep (5) and Gordon *et al.* (12) reported that the

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quality as well as the quantity of dietary protein were important in the weight response of rats to growth hormone. Follis (11) stated that growth hormone failed to promote growth in hypophysectomized rats whose dietary intake was restricted. On the other hand, Quimby *et al.* (20), working with rats recovering from inanition, and Marx *et al.* (18), using hypophysectomized rats, found that adequate amounts of growth hormone caused increased tissue deposition. The present report describes observations made on phenylalanine-tyrosine-deficient rats and their pair-fed controls when treated with exogenous growth hormone.

Material and Method

Forty-six Sprague-Dawley male rats, averaging 70 grams, were divided into three groups. One group of six rats (normal control) was fed a purified diet consisting of 19 crystalline amino acids, vitamins, sucrose, cottonseed oil and the necessary minerals as described by Rose *et al.* (22). A second group of 20 rats were fed the same diet, complete in every respect, except for the total omission of phenylalanine and tyrosine (deficient). The caloric value of the missing amino acids was supplied by additional sucrose. The third group was pair-fed the complete diet so that the consumption of food of each was no greater than that of its deficient partner. All rats were kept in individual cages and, with the exception of the pair-fed group, were fed *ad libitum*. Water was readily available to all rats.

One-half of the normal control and deficient rats, with their pair-fed partners, was selected to receive a daily intraperitoneal injection of sterile growth hormone** for 30 days. The hormone was dissolved in sterile physiological saline and a dose of 0.5 ml of this solution was calculated as containing 1.0 mg of hormone. This was injected in the amount of 1.0 mg per 100 grams of body weight, per day. The rats of each group which did not receive growth hormone were injected daily with 0.5 ml of saline solution per 100 grams of body weight.

Each animal was weighed daily before feeding. The amount of food consumed each day by each deficient rat was measured and used as a basis for paired feeding. After 30 experimental days, the rats were killed by exsanguination and both tibias and a segment of 3-4 thoracic vertebrae were removed and fixed in neutral formalin. One tibia was cut transversely and the proximal portion split on a mid-

** The growth hormone was purchased from Armour Laboratories, Lot No. AL-1032.

sagittal plane and stained by the silver nitrate method of Evans *et al.* (10). Using this piece, the width of the epiphyseal cartilages was measured on a compound microscope equipped with a calibrated 10X measuring eyepiece and a 5.8X objective. Eight to 12 readings were taken across each epiphyseal plate and the average computed. The other tibia and the vertebrae were decalcified, paraffin embedded, sectioned and stained with hematoxylin and eosin.

The average daily body weight of each group of rats was calculated from the individual weights. The average weight of diet consumed during the 30-day period was calculated for the growth hormone-treated and saline-treated deficient rats. The amount of food consumed by each of these groups was compared with the other to determine whether or not there was a significant difference.

Observations

The growth hormone-treated deficient rats consumed a significantly greater amount of food than did the saline-treated deficients; the former eating an average of 3.41 ± .24 grams per day, the latter 2.97 ± .17 grams per day. The "t" value for this difference was computed to be 5.74 with a confidence level of .001. The food intake of the normal controls was not measured, but, at the close of the experiment, each animal, whether receiving growth hormone or not, was eating about 11-12 grams of food per day.

There was no significant difference in the average weight gain between the normal control rats which received growth hormone and those which were treated with saline (Fig. 1). Likewise, growth hormone had no significant effect on the body weights of those rats deprived of phenylalanine and tyrosine, even though their mean consumption of food was significantly greater than in the saline-treated deficients. Both groups continued to lose weight throughout the experiment (Fig. 2). The mean body weight of the growth hormone-treated deficients dropped from 68.3 ± 2.0 grams to 46.4 ± 1.5 grams, while the mean body weight of the saline-treated deficients went from 68.4 ± 2.1 grams to 43.9 ± 1.4 grams during the same period. An entirely different picture was seen when the mean body weights of the two pair-fed groups were compared (Fig. 2). The growth hormone-treated pair-fed rats (whose diet was hypocaloric, being restricted in amount to that consumed each day by the growth hormone-treated deficients) lost an average of 13 ± 1.5 grams from the initial

experimental day until about the 12th day at which time the average body weight of the group increased and, by the end of the 30-day period, their mean body weight was 70.1 ± 2.2 grams, or only 5 grams less than the mean weight on day 1. The saline-treated pair-fed rats averaged 72.6 ± 2.3 grams initially and dropped to 56.6 ± 1.4 grams finally.

The mean widths of the proximal tibial epiphyseal disks of the saline-treated and of the growth hormone-treated deficient groups did

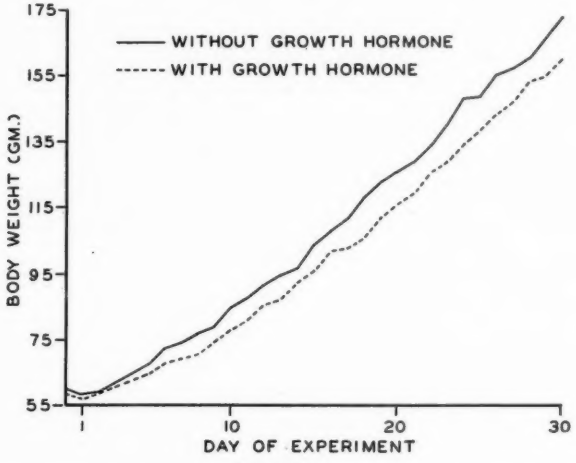

FIGURE 1
Mean body weight curves of normal control rats with and without growth hormone administration.

not differ significantly, being 58 ± 7 micra in the former and 62 ± 17 micra in the latter. The tibial epiphyseal plates of the growth hormone-treated pair-fed rats averaged 151 ± 16 micra and those of the saline-treated pair-fed rats averaged 79 ± 15 micra. The level of confidence when comparing these two mean measurements is .001. The tibial epiphyseal widths of the normal control rats, with or without growth hormone, were not significantly different.

Histology. Normal Control. There were no important differences in histological appearance between the tibial or vertebral epiphyseal plates of the growth hormone-treated or saline-treated normal control rats. The proximal tibial epiphyseal cartilage showed the well-

known zones of active chondrogenesis and osteogenesis (Figs. 3, 4). The columns of chondrocytes were relatively straight with an orderly division into zones of reserve, proliferating, hypertrophic and degenerating cells. Large, active osteoblasts covered the bony trabeculae

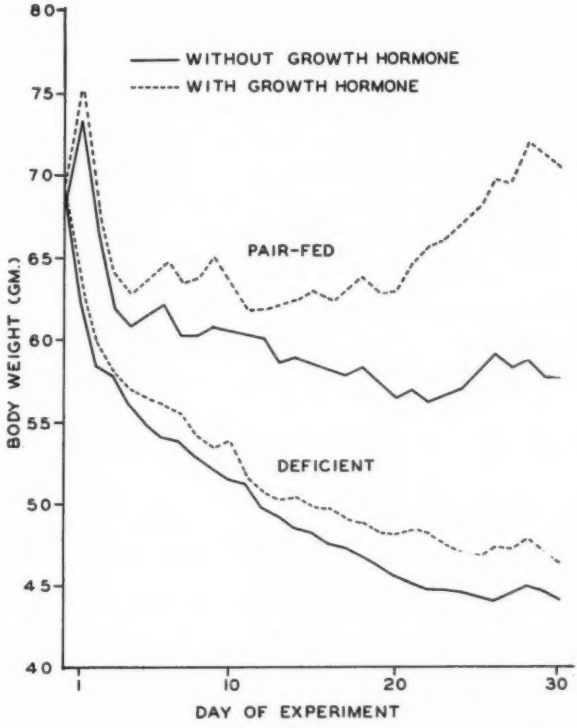

FIGURE 2

Mean body weight curves of phenylalanine-tyrosine-deficient rats and their pair-fed controls with and without growth hormone.

on the metaphysis side of the cartilage. Capillary erosion into the degenerating columns was easily seen and long, bony trabeculae formed a dense primary spongiosa in the metaphysis.

The vertebral epiphyseal cartilage showed activity (Figs. 9, 10). The columns of chondrocytes were regular but smaller and the car-

FIGURE 3

Proximal tibial epiphyseal cartilage of a saline-treated normal control rat. X70.

FIGURE 4

Proximal tibial epiphyseal cartilage of a growth hormone-treated normal control rat. X70.

FIGURE 5

Proximal tibial epiphyseal cartilage of a saline-treated pair-fed control rat. X70.

FIGURE 6

Proximal tibial epiphyseal cartilage of a growth hormone-treated pair-fed control rat. X70.

FIGURE 7

Proximal tibial epiphyseal cartilage of a saline-treated phenylalanine-tyrosine-deficient rat. X70.

FIGURE 8

Proximal tibial epiphyseal cartilage of a growth hormone-treated phenylalanine-tyrosine-deficient rat. X70.

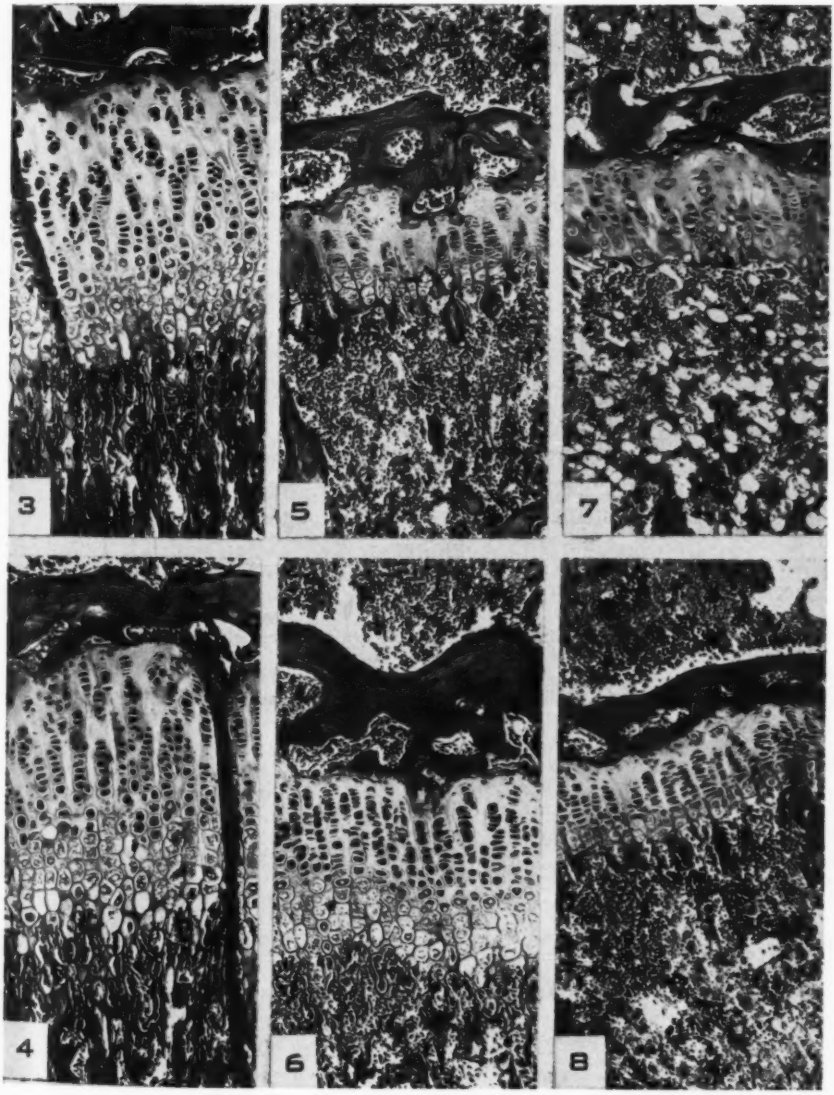
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FIGURE 9

Vertebral epiphyseal cartilage of a saline-treated normal control rat. X70.

FIGURE 10

Vertebral epiphyseal cartilage of a growth hormone-treated normal control rat. X70.

FIGURE 11

Vertebral epiphyseal cartilage of a saline-treated pair-fed control rat. X70.

FIGURE 12

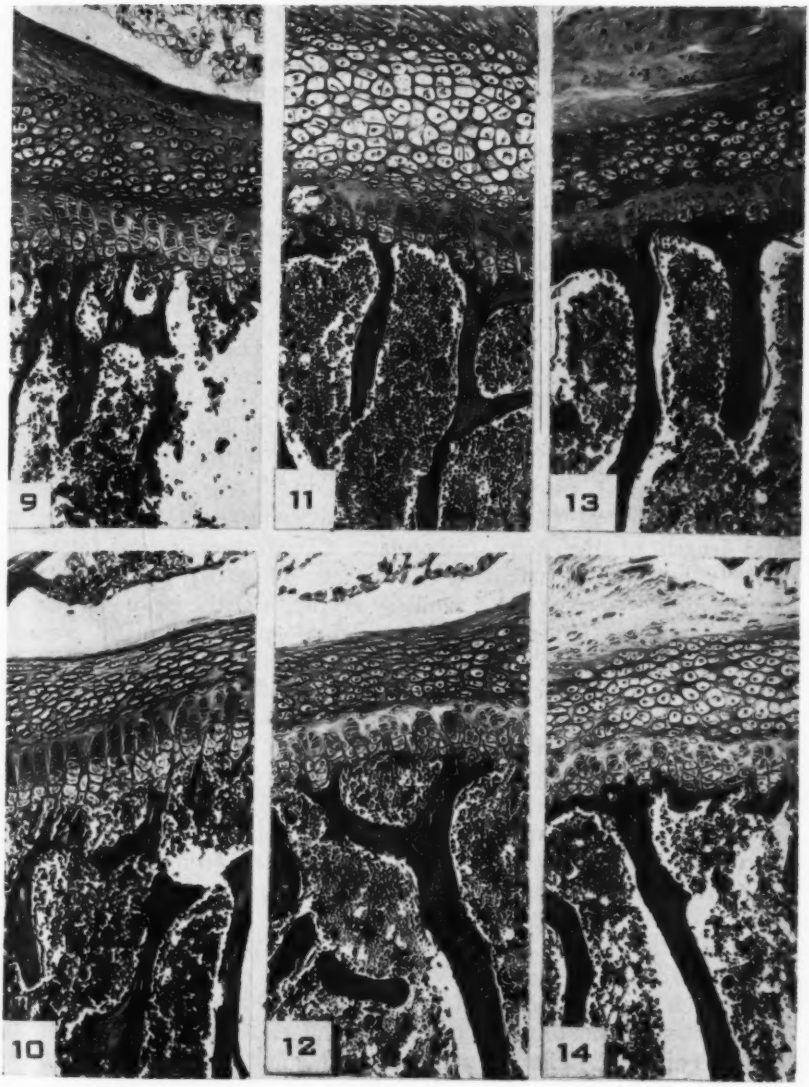
Vertebral epiphyseal cartilage of a growth hormone-treated pair-fed control rat. X70.

FIGURE 13

Vertebral epiphyseal cartilage of a saline-treated phenylalanine-tyrosine-deficient rat. X70.

FIGURE 14

Vertebral epiphyseal cartilage of a growth hormone-treated phenylalanine-tyrosine-deficient rat. X70.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tilage plate was thinner than in the tibial epiphysis. The bony trabeculae were thicker than those in the tibia.

Pair-fed control: saline-treated. When compared to the normal control rats, the proximal tibial epiphyseal cartilage plates of these rats showed a light eosinophilic matrix separating somewhat irregular, compressed columns of small chondrocytes (Fig. 5). The metaphyseal region contained fewer and shorter bony trabeculae than normal. The vertebral epiphyseal plates appeared to be slightly narrower than normal, but these were not measured. They showed little cellular activity, decreased vascularity and little bony replacement. A very thin plate of bone sealed off the epiphyseal cartilage from the marrow cavity in most of these rats (Fig. 11).

Pair-fed control: growth hormone-treated. The tibial epiphyseal plates of these rats, although narrower than normal, compared very favorably with them histologically. The chondrocytes were small, but were arranged in long, straight, well organized columns and the zones associated with normal activity were easily discernible. Compared to the saline-treated, there was increased capillary erosion, an increase in the number of chondrocytes and an increase in the number and length of the bony trabeculae. Chondrogenesis and osteogenesis were very active in these rats (Fig. 6). The vertebral epiphyses showed more capillary erosion and the bony cap was not in evidence. Otherwise, these vertebral epiphyseal plates were not much different than those of the saline-treated rats (Fig. 12).

Deficient: saline-treated. The proximal tibial epiphyseal plates of these rats showed no chondrogenic or osteogenic activity. The columns of chondrocytes were disorganized and very irregular. The region of the metaphysis adjacent to the epiphyseal cartilage contained no bony trabeculae. The usual regions of epiphyseal cartilage activity were not apparent (Fig. 7). The vertebral epiphyseal plates were narrow. The most prominent feature in these structures was the presence of a thick, dense bony cap sealing the narrow cavity from the epiphyseal plate (Fig. 13).

Deficient: growth hormone-treated. The proximal tibial epiphyseal cartilage of these rats were, in general, no wider than in the rats which received saline. However, in the growth hormone-treated the number of chondrocytes was greatly increased and arranged into columns and zones characteristic of active epiphyseal cartilage. The metaphyseal bony trabeculae were numerous (Fig. 8). The verte-

bral epiphyseal plates of these rats were practically identical with those of the saline-treated rats with one exception. The bony cap, which separated the epiphyseal plate and the marrow cavity, was considerably thinner (Fig. 14).

Discussion

The lack of effect of growth hormone on body weight increment or endochondral bone growth in the normal control rats is difficult to explain. Evans and Long (9) described a greater rate of increase in body weight of growth hormone-treated normal rats than in their untreated litter-mate controls. The rate of divergence between the body weights of these two groups increased with continued treatment with advancing age. Ray *et al.* (21) reported that the administration of growth hormone to normal 54-day-old rats was followed by a slight increase in the width of the epiphyseal cartilage, but not in sufficient amount to be significant. In the present experiment, it is possible that the normal control rats were producing sufficient endogenous growth hormone to maximally stimulate their growth potential and the addition of exogenous growth hormone was, therefore, without effect.

Chow and Greep (5) demonstrated that the amount of weight gain induced by growth hormone was dependent upon the quality of protein fed to rats. This conclusion is substantiated by us with regard to weight gain, since our deficient rats failed to hold their weight even when receiving growth hormone. However, the fact that weight increment was not produced in these growth hormone-treated deficient rats does not necessarily indicate that growth hormone was without effect in such rats. The tibial and vertebral epiphyseal cartilages of these animals showed evidence of resuming activity, even though the rats constantly lost body weight.

Bavetta *et al.* (2) observed a lack of weight increment in growth hormone-treated rats fed a tryptophan-low diet. These investigators also observed that the proximal tibial epiphyseal plates of such rats were increased in width and that chondrogenesis and osteogenesis were resumed, although the animals consumed no more food than deficient rats which received saline only.

Ershoff (7) has emphasized the possibility that there may be a decreased secretion of pituitary growth hormone during inanition. McHenry (19) found, in vitamin B-6 studies, that growth hormone did not cause weight increase in pair-fed control rats if a nutritional

deficiency was caused by a drastic reduction in caloric intake of the control diet to that of the deficient level. The growth hormone-treated pair-fed rats in our experiment certainly received a calorically inadequate diet, yet, although they experienced a weight loss in the early part of the experiment, they gained considerably in the latter two-thirds of the experiment and lost very little weight when considered over the entire experimental period. In addition, the histological pictures of their tibial epiphyseal cartilages showed evidence of response to growth hormone stimulation. The reversal of body weight loss to one of gain after 12 days of treatment with growth hormone in these pair-fed rats is without explanation, other than that it was induced by exogenous growth hormone.

The continued loss of weight in the growth hormone-treated deficient rats suggests a decreased sensitivity of their tissues, in general, to this hormone. The increased cellularity and activity of their epiphyseal disks indicates that the metabolic processes involved with bone growth were not insensitive to growth hormone stimulation in spite of the amino acid deficiencies.

Weil (31 and Li (15) have pointed up the role of growth hormone in intermediary metabolism. Russell (23) has shown that one of the effects of growth hormone is to increase the formation of intracellular protein by accelerating the conversion of amino acids to protein. Samuels (24) has stated that the pituitary hormones have a regulatory part in protein anabolism. Quimby *et al.* (20) reported that rats recovering from inanition maintained a weight gain equal to that of the controls when injected with large doses of growth hormone. The studies of Marx *et al.* (18) and Li *et al.* (16) showed that growth hormone caused increased tissue protein through better utilization of consumed food in hypophysectomized rats. The marked recovery of weight and the intense stimulation of the tibial epiphyseal plates of our growth hormone-treated pair-fed rats resulted, we believe, from a more efficient use of ingested food.

The less prominent changes which occurred in the vertebral epiphyses of the growth hormone-treated deficient and pair-fed rats, when compared to the response of the proximal tibial epiphyses, could be expected when one considers that the vertebrae are normally slower growing than the tibias. Also, vertebral growth is accomplished from two epiphyses of approximately equal size, while increase in length of the tibias occurs from the proximal epiphysis primarily (1).

Summary and Conclusions

1. Growth hormone administration to young male phenylalanine-tyrosine deficient rats resulted in some stimulation of the proximal tibial epiphyseal cartilage and some reversal of bony plate deposition in the vertebrae, but had no effect on body weight loss.

2. Administration of growth hormone to pair-fed rats (on a hypocaloric diet) resulted in substantial weight increment after an initial weight loss and marked stimulation of the proximal tibial epiphyseal cartilage plates.

3. Growth hormone retained its ability to stimulate appetite in rats fed a phenylalanine-tyrosine-deficient diet.

4. The secretion of endogenous growth hormone is more seriously inhibited by the total lack of phenylalanine and tyrosine than by hypocaloric feeding.

5. The response of the proximal tibial epiphyseal cartilage is a more sensitive measure of growth hormone stimulation than body weight increment.

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